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CHICKEN HEPATIC METABOLISM *IN VITRO*. PROTEIN AND ENERGY RELATIONS IN THE BROILER CHICKEN—VI. EFFECT OF DIETARY PROTEIN AND ENERGY RESTRICTIONS ON *IN VITRO* CARBOHYDRATE AND LIPID METABOLISM AND METABOLIC HORMONE PROFILES*

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Abstract—1. Ross male broiler chicks growing from 14 to 28 days of age were fed 14 and 20% protein diets (4 kcal day^{-1}/body $wt^{0.66}$) or 20 and 28% protein diets (2.8 kcal day^{-1}/body $wt^{0.66}$) in a 2 × 2 factorial arrangement to determine the effects of protein and energy intakes on *in vitro* lipogenesis (IVL) and net glucose production (NGP). Plasma concentrations of insulin, glucagon, thyroid hormones (T_3 and T_4) and somatomedin-C (Sm-C) were estimated by radioimmunoassay.

2. There was a significant ($P < 0.05$) decrease in IVL in the chicks given the higher daily protein intake.

3. The higher protein intake increased ($P < 0.05$) NGP while the lower energy intake decreased ($P < 0.05$) NGP.

4. Insulin, both thyroid hormones and Sm-C were affected by dietary energy and protein intakes.

INTRODUCTION

Although the level of dietary protein and of limiting amino acids influence both lean and adipose tissue development in broiler chickens, interpretation of effects can be confounded by dietary energy intake. Interpretation of the effects of both dietary carbohydrate and protein on lipogenesis in chickens is further complicated, even in isocaloric diets, by difficulties in dietary formulations. For example, an increase in dietary protein is normally accompanied by a decrease in the dietary carbohydrate level. The importance of dietary carbohydrate was illustrated by Clark *et al.* (1979) who found little relationship between the level of the dietary fat and *de novo* lipogenesis when chicks were provided the same amount of dietary carbohydrate in all dietary treatments. In contrast, the role of dietary protein in the regulation of lipid metabolism in the chick appears to be more complex than that of dietary fat. For example, Yeh and Leveille (1969) and Rosebrough *et al.* (1986) reported that an improvement in dietary protein quality (addition of the limiting amino acid lysine to a low protein diet) depressed *de novo* lipogenesis in chicks. Confounding the role of amino acid supply as a regulator of lipogenesis in all birds was the observation that turkey poults overeat diets containing a wide calorie:protein ratio (> 17:1), even when these diets are supplemented with limiting amino acids to meet requirements (NRC, 1984). The net effect is still an increase in lipogenesis even in the presence of an ideal dietary amino acid profile.

Thus, in defining the mechanisms surrounding the role of dietary protein in avian lipid metabolism, one must look farther than just meeting the amino acid requirement for a particular stage of growth. The liver is the main site of *de novo* lipid synthesis in birds as well as a major point of degradation and transamination of dietary amino acids. We have found active net synthesis of glucose (gluconeogenesis) in chicks fed high-protein diets (> 23%) and have speculated that carbon skeleton rearrangement during gluconeogenesis directed substrates from lipid synthetic pathways and required energy which could be used for lipogenesis. We are unaware of any work describing gluconeogenesis, lipogenesis and endocrine function under defined energy and protein intakes because some form of limited feeding regime is necessary to separate the effects of dietary protein and energy intake. The experiments described in this report were designed to test the effects of absolute protein and energy intakes on avian carbohydrate and lipid metabolism.

MATERIALS AND METHODS

Animals

Fourteen-day-old Ross male broiler chicks (average wt 300 ± 8 g) were fed 14 and 20% protein diets (4 kcal day^{-1}/body $wt^{0.66}$) or 20 and 28% protein diets (2.8 kcal day^{-1}/body $wt^{0.66}$) in a 2 × 2 factorial arrangement (eight pen replicates of six chicks per dietary treatment) to determine the independent effects of dietary protein and energy intakes. A preliminary experiment indicated that the first formula described median daily voluntary feed intake of chicks growing from 14 to 35 days of age. The diet

*Mention of a trade name, proprietary product or vendor does not constitute a guarantee or warranty of the product by USDA or imply its approval to the exclusion of other suitable products or vendors.

Table 1. Composition of diets

Ingredient	Dietary protein (%)		
	14	20	28
	(g/kg)	(g/kg)	(g/kg)
Corn meal	700	700	700
Soybean meal	170	100	
Soy protein*		100	240
Corn oil	30	20	
Sand	35	15	
Methionine	5	5	
Dicalcium phosphate	40	40	40
Limestone	10	10	10
Selenium premix†	1	1	1
Mineral premix†	1	1	1
Vitamin premix†	5	5	5
Iodized salt	3	3	3

*Alpha Protein: Nutritional Biochemicals, Cleveland, OH.
†For a description of these premixes see Rosebrough *et al.* (1985).

formulations are shown in Table 1. Chicks were weighed every 3 days and feed intake adjusted according to growth of the group fed the 20% protein diet at 4 kcal day^{-1}/body $wt^{0.66}$. Chicks were kept in electrically heated battery-brooders with raised wire floors and allowed water on an *ad libitum* basis. The room temperature was maintained at 25°C and a 12 hr light cycle was used (06.00 to 18.00 hr).

In vitro *metabolic studies*

General. Chicks were randomly selected from each pen at 28 days of age and bled via cardiac puncture into combination syringe-plasma collection tubes (Sarstedt Corp., Princeton, NJ). Chicks were selected from each pen at 09.00 hr to minimize possible diurnal variation in metabolite and hormone levels. The chicks were killed by cervical dislocation, weighed and the livers were rapidly removed, weighed, and placed in individual vessels containing 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid)–155 mM NaCl (pH 7.5). Blood was centrifuged at 600 *g* for 30 min and the plasma was then drawn off with an individual Pasteur pipette and stored at −70°C until analyzed for metabolite concentrations.

Enzyme activities. A portion of each liver was homogenized in 50 mM HEPES–3.3 mM mercaptoethanol (pH 7.5) and centrifuged for 60 min at 50,000 *g*. The supernatant was frozen at −80°C until analyzed for the activities of malic enzyme (ME), tyrosine aminotransferase (TAT), NADP-isocitrate dehydrogenase (ICD), fatty acid synthetase (FAS) and lysine:alpha ketoglutarate reductase (LAKGR). Enzyme activities were determined according to our established procedures (Rosebrough and Steele, 1985) and expressed as micromoles of product formed per minute under the assay conditions.

Lipogenesis. Another portion of each liver was sliced (50–75 mg) with a Stadie-Riggs hand microtome. Duplicate slices were incubated at 37°C for 2 hr in Hanks' balanced salts (HBSS; Hanks and Wallace, 1949) supplemented with 10 mM HEPES, 1% bovine serum albumin and either 10 mM [2-^{14}C] sodium acetate or 10 mM [2-^{14}C] pyruvate (37 DPM/nmol). The slices were then extracted and treated (Rosebrough and Steele, 1985).

Glucose production. Duplicate slices were incubated at 37°C for 2 hr in HBSS supplemented with 10 mM HEPES, 1% bovine serum albumin in the presence and absence of 10 mM pyruvate. Glucose was then measured in the medium by using a coupled hexokinase + glucose-6-phosphate dehydrogenase reaction (Stein, 1963). An aliquot of medium was also passed over an ion exchange column (BioRad AG 1-X8) to separate glucose from polar metabolites. The eluate was then analyzed for both glucose concentration and precursor count incorporation into glucose.

Plasma metabolites. Plasma insulin concentration was estimated with a homologous avian radioimmunoassay system which uses chicken insulin as both the standard and ^{125}I-labeled tracer and a primary antisera raised against chicken insulin (McMurtry *et al.* 1983). Both T_3 and T_4 were estimated with commercially available kits (Immuchem Corp., Carson, CA). Apparent somatomedin-C (Sm-C) was estimated by slightly modifying the method of Huybrechts *et al.* (1985) by using a human Sm-C radioimmunoassay (Nichols Diagnostics, San Juan Capistrano, CA). The assay was validated by noting parallelism of graded amounts of a pool of chicken plasma (Fig. 1) and recovery of a known quantity of human Sm-C (Amgen) from graded amounts of the plasma pool. Values for Sm-C were calculated on the basis of units per ml and ng/ml.

Statistical analyses. Metabolic activities are expressed on the basis of relative liver size of the chick (liver as per cent of body wt × metabolic activity per g liver) to account for changes in metabolic activity relative to independent changes in liver size. Values for chicks from a particular pen were averaged to derive a pen mean because we considered the pen of chicks subjected to a particular treatment as the experimental unit. Significance of differences between treatment means was determined at a 0.05 level of probability. Because of an initial lack of homogeneity of error variances for enzyme activities, data were subjected to natural log transformations to develop pooled estimates of error variances. The data were analyzed as a 2 × 2 factorial arrangement of treatments. Terms in the model for the analyses of variance were protein and energy intakes (Remington and Schork, 1970).

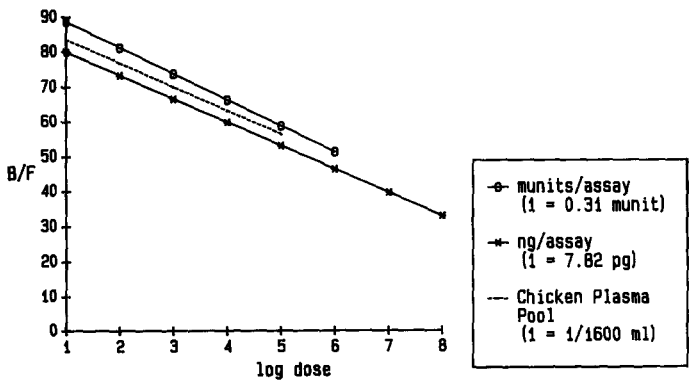

Fig. 1. Least squares fit for the validation of the use of a human Sm-C assay for chicken samples.

Table 2. Effect of restricted energy or protein intakes on chick growth and feed consumption from 14 to 28 days of age (values presented are means of eight pen means per dietary treatment)

Parameter	Dietary protein (%)				Significant main treatment effects* ($P < 0.05$)
	14%	20%R	20%	28%R	
Body weight 28 days (g)	802	720	940	772	Pro, E
Feed 14–28 days (g)	1087	762	1087	762	
Apparent feed efficiency (F/G)	2.1	1.8	1.7	1.6	E
Backskin fat (%)	67.6	55.7	55.8	45.7	Pro, E
Breast muscle (g/100 g body wt)	8.6	10.5	9.9	11.7	Pro, E
Liver size (g/100 g body wt)	3.0	2.8	2.8	2.5	

*Determined from an analysis of variance: Pro = protein intake (14% and 20%R = low protein intake, 20% and 28%R = high protein intake); E = energy intake (20%R and 28%R = low energy intake, 14% and 20% = high energy intake).

RESULTS

Growth and development

Both higher protein (20% + 28%R vs 14% + 20%R) and energy intake (14% + 20% vs 20R + 28%R) resulted in greater ($P < 0.05$) body wts at 28 days of age (Table 2). In contrast, apparent efficiency of feed utilization was poorer ($P < 0.05$) in chicks given the higher energy intake. Backskin fat was used as an indicator of carcass fat stores and was greatest ($P < 0.05$) in the chicks fed a low protein intake coupled to the high energy intake (14%) and least ($P < 0.05$) in chicks fed the high protein intake coupled with the low energy intake (28%R). Relative breast muscle size followed the opposite, though significant ($P < 0.05$), trend. Although relative liver size was not significantly affected by any of the dietary treatments, expressing metabolic activities on this basis reduced overall variation in most of the analyses of variance.

Plasma metabolites

Table 3 shows the combined effects of dietary energy and protein intakes on several plasma hormones and metabolites that have been implicated in the regulation of carbohydrate and lipid metabolism. There was a positive effect ($P < 0.05$) of both energy restriction and lower dietary protein on plasma insulin but no effect of either on plasma glucagon. In contrast, energy restriction decreased ($P < 0.05$) plasma T_3 and triglycerides while higher protein increased ($P < 0.05$) T_4. Plasma Sm-C, whether expressed on the basis of activity units or mass, was increased ($P < 0.05$) by both energy restriction (20%R + 28% vs 14% + 20%) and by an increase in protein (20% + 28R% vs 14% + 20%R).

Figure 1 illustrates the method used to validate the use of a human Sm-C assay to estimate concentrations in chickens. The data reveal a highly significant ($R = 0.98$; $P < 0.001$) relationship between concentrations attained by using a human activity standard series and by using recombinant human Sm-C standard series (Amgen). Plasma glucose was not changed by any of the dietary treatments.

In vitro *lipogenesis and glucose production*

In the context of this experiment (limited dietary energy intake in all treatment groups and thus no true *ad libitum* feeding regime), we found that an increase

Table 3. Effect of restricted energy or protein intakes on certain plasma metabolic hormone and metabolite concentrations in 28-day-old chicks (values presented are means of eight pen means per dietary treatment)

Parameter	Dietary protein (%)				Significant main treatment effects* ($P < 0.05$)
	14%	20%R	20%	28%R	
Insulin (pg/ml)	213	350	161	250	Pro, E
Glucagon (pg/ml)	566	636	658		
Insulin/Glucagon	0.4	0.6	0.2	0.4	
T_3 (ng/ml)	6.1	4.1	5.9	4.1	E
T_4 (ng/ml)	5.2	6.7	7.2	7.8	Pro
Somatomedin-C					
units/ml	0.9	1.3	1.4	1.7	Pro, E
ng/ml	13.5	18.2	19.1	24.3	Pro, E
Triglycerides (mg/100 ml)	98.1	76.3	95.4	71.1	E
Glucose (mg/100 ml)	273	255	255	265	

*Determined from an analysis of variance: Pro = protein intake (14% and 20%R = low protein intake, 20% and 28%R = high protein intake); E = energy intake (20%R and 28%R = low energy intake, 14% and 20% = high energy intake).

Table 4. Effect of restricted energy or protein intakes on *in vitro* lipogenesis and certain lipogenic and gluconeogenic enzyme activities in 28-day-old chicks (values presented are means of eight pen means per dietary treatment)

	Dietary protein (%)				Significant main treatment effects*
	14%	20%R	20%	28%R	($P < 0.05$)
Lipogenesis† in the presence of acetate or pyruvate (μmol/100 g body wt)					
[2-^{14}C]Acetate	104	89	73	57	Pro
[2-^{14}C]Pyruvate	185	135	160	121	Pro
Lipogenesis† in the presence of both acetate and pyruvate (μmoles/100 g body wt)					
Acetate + pyruvate	246	154	161	129	Pro, E
[2-^{14}C]Acetate	96	20	47	29	Pro, E
[2-^{14}C]Pyruvate	150	134	114	100	Pro
Enzyme activities‡ (units/100 g body wt)					
NADP-ICD	10.6	14.2	14.7	20.1	Pro
ME	8.6	7.3	7.2	4.6	Pro, E
FAS	5.6	2.2	4.8	2.1	E
TAT	5.5	5.2	5.4	5.0	
LAKGR	140.0	141.5	149.5	166.8	

*Determined from an analysis of variance: Pro = protein intake (14% and 20%R = low protein intake, 20% and 28%R = high protein intake); E = energy intake (20%R and 28%R = low energy intake, 14% and 20% = high energy intake).

†Conditions for the assay are described in Materials and Methods. Expressed as μmol of the particular substrate used per 100 g body wt.

‡Conditions for the determination of enzyme activities are described in Materials and Methods; NADP-ICD, nicotinamide adenine dinucleotide-dependent isocitrate dehydrogenase; ME, malic enzyme; FAS, fatty acid synthetase; TAT, tyrosine aminotransferase; LAKGR, lysine:alpha ketoglutarate reductase.

in protein intake decreased ($P < 0.05$) *de novo* lipogenesis from both acetate and pyruvate (Table 4). A comparison of the use of pyruvate and acetate revealed that pyruvate utilization exceeded that for acetate although total acetyl group utilization (acetate + pyruvate) followed the previously described dietary trend noted for acetate utilization. Analysis of enzyme activities revealed that both an increase in protein and a decrease in energy intake decreased ($P < 0.05$) ME activity. The higher protein intake increased ICD activity and the higher energy intake increased FAS activity.

In contrast to the above noted effects of dietary treatments on substrate utilization for lipogenesis, basal glucose production was increased (Table 5; $P < 0.05$) by an increase in dietary protein (20% and 28%R vs 14% + 20%R) while net production in the presence of pyruvate was increased by energy restriction (20%R + 28R% vs 14% + 20%). The activities of TAT and LAKGR were unaffected by either protein or energy intake.

DISCUSSION

Prior work from our laboratory, as well as that from other groups, suggest that dietary protein intake *per se* is a potent regulator of *de novo* lipid metabolism in birds. Most of these studies have centered around feeding methods that either mimic fasting–refeeding regimes or involve forced-feeding of protein in conjunction with graded amounts of carbohydrates. In both situations, results are specific only for the amount of diet consumed during refeeding or forced-feeding episodes. Both methods of altering protein or energy intake involve variation in normal

Table 5. Comparison of net glucose production and the relative use of either pyruvate or acetate as *in vitro* glucose substrates in chicks fed restricted energy or protein intake from 14 to 28 days of age (values presented are means of eight pen means per dietary treatment)

	Dietary protein (%)				Significant main treatment effects*
	14%	20%R	20%	28%R	($P < 0.05$)
Net glucose production† (μmol/100 g body wt)					
	62	63	95	124	Pro
Specific substrate utilization for glucose production‡ (μmol/100 g body wt)					
[2-^{14}C]Acetate	31	63	39	73	
[2-^{14}C]Pyruvate	173	260	296	421	Pro, E
Acetate + pyruvate					
[2-^{14}C]Acetate	19	29	33	88	
[2-^{14}C]Pyruvate	217	312	333	495	Pro, E

*Determined from an analysis of variance: Pro = protein intake (14% and 20%R = low protein intake, 20% and 28%R = high protein intake); E = energy intake (20%R and 28%R = low energy intake, 14% and 20% = high energy intake).

†Assay conditions are described in Materials and Methods. Expressed as μmol of glucose produced per 100 g body wt.

‡Assay conditions are described in Materials and Methods. Expressed as μmol of the particular substrate used for glucose production per 100 g body wt.

feeding behavior which must be considered in interpreting results.

The results of the present study support those of Tanaka *et al.* (1983) which indicates that in the face of a constant dietary carbohydrate intake, an increase in protein calories will result in a decrease in lipogenic enzyme activities. The results of the present study further indicate that the effect of dietary protein can be separated from that of dietary energy. Although ME may provide the necessary NADPH for lipogenesis, the enzyme may not strictly regulate lipogenesis according to the data in the present study. For example, the lowest noted activity (28% protein fed at 30% restriction) would result in the formation of nearly 5 times the NADPH required for the *de novo* synthesis of the fatty acids noted for this group (Table 4). A more plausible explanation is that ME reflects NADPH utilization and does not, *per se*, regulate fatty acid synthesis.

Few groups have offered any biochemical logic for the decrease in lipogenesis accompanying the feeding of high-protein diets. Yeh and Leveille (1969) found an inverse relationship between the level of the dietary protein and the subsequent rate of *in vitro* lipogenesis and speculated that an increase in the dietary protein level decreased the flow of substrates through glycolysis and increased the production of glucose from substrates that were formerly in the pathways leading to fat synthesis. Yeh and Leveille (1969) also compared slopes in varied protein, isocarbohydrate and varied carbohydrate dietary treatment groups and found slight differences in favor of protein as a regulator of lipogenesis. The enzyme activities in this study also suggest that isocitrate dehydrogenase may function in both lipid and protein metabolism by providing a residual capacity for the production of reducing equivalents during a period of decreased malic enzyme activity. Intracellular competition may exist between acetyl CoA carboxylase and the aconitase–isocitrate dehydrogenase pathway for limited cytoplasmic citrate. Thus, the requirement for alpha ketoglutarate (a product of the reaction catalyzed by isocitrate dehydrogenase) as a co-reactant for transamination, which occurs during a time of increased dietary protein intake, would depress citrate levels to a point that activation of acetyl CoA carboxylase would not occur. Indeed, Clark *et al.* (1979) reported that avian acetyl CoA carboxylase was not controlled by phosphorylation–dephosphorylation as was the rat enzyme but was particularly sensitive to citrate levels. The latter report seems to offer some support for the role of high-protein diets as regulators of lipogenesis via citrate availability.

The apparent decrease in lipogenesis from acetate in the presence of pyruvate and from pyruvate in the presence of acetate suggests that both compete for limited mitochondrial coenzyme A and that the apparent decrease, in each case, reflected pool dilution by the unlabeled precursor rather than inhibition. This hypothesis seems to be supported by corresponding values for total acetyl equivalent utilization. The finding of the superiority of pyruvate as a lipogenic substrate casts doubt on the separation of gluconeogenic and lipogenic pathways in chick liver. Pyruvate carboxylase, commonly thought of as a key enzyme in gluconeogenesis, catalyzes the formation of mitochondrial oxaloacetate from pyruvate and CO_2. At this point, oxaloacetate could condense with acetyl coenzyme A to form citrate or be a source of phosphoenolpyruvate for the production of glucose. The transport of citrate across the mitochondrion provides lipogenic substrates and requires that oxaloacetate be replenished. Thus, the carboxylation of pyruvate ultimately results in the provision of cytoplasmic reducing equivalents as well as the actual carbon skeleton required for the *de novo* synthesis of lipids.

Results of work with teoelosts suggest that amino acids, rather than glucose, are the major stimulus for insulin release from the islet cells. Ablett *et al.* (1983) proposed that high-protein diets chronically elevate insulin in salmon and result in reduced insulin binding by liver and muscle membrane preparations. The subsequent down regulation of the insulin receptor by elevated circulating insulin was similar to responses noted in mammals and may also explain the apparent insulin insensitivity noted in birds. It is possible that tests of insulin sensitivity in birds may also require the consideration of the feeding regime utilized, the level of the dietary protein and the availability of certain amino acids.

In comparing Sm-C concentrations among animal species, lower concentrations were noted in the chicken (Leung *et al.*, 1986) than in the growing rat (Prewitt *et al.* 1982) although trends associated with growth are similar in both species. Prewitt *et al.* (1982) also noted a linear increase in plasma Sm-C with increasing protein intake coupled to a moderate energy restriction (75% of *ad libitum*). It is tempting to speculate that the increase in muscularity (increase in relative breast muscle size) in chickens given a higher daily protein allotment may relate to Sm-C. Growth retardation in chickens may also be due to reduced Sm-C as well as increased catabolic hormones. Buyse *et al.* (1986) used exogenous corticosterone to reduce growth and increase body fat of chickens and found a decrease in plasma Sm-C concentration. Huybrechts *et al.* (1985) surveyed Sm-C concentrations in growing meat and egg-type chickens and found an age-dependent decrease in Sm-C in the egg-type but not in the meat-type chicken. The latter observation is not surprising because the meat-type chicken has been intensely selected for rapid growth. A recent paper (Lauterio and Scanes, 1987) compared Sm-C concentrations in the same strains of chickens as a function of dietary protein and showed a decrease in Sm-C when chicks were switched from a 20% to a 5% protein diet. This change in Sm-C was attributed to changes in protein nutritional status. An examination of feed intakes in the latter study case some doubt on this hypothesis because feed intake was not controlled as in the present study. Thus, protein intake *per se* cannot be separated from the energy intake. We have no explanation for the apparent difference in feed intake when our control group (20% fed at 4 kcal day^{-1}/body $wt^{0.66}$) is compared to that of Lauterio and Scanes (77 g/day vs 32.2 g/day for the same age period and for a similar diet, 14–28 days) or for the observation that our restricted broiler chicks were also heavier than were the control broiler chicks in the latter

study. The present study, using growing chickens, was designed to test the relationship between protein and energy intakes and various indices of growth. Plasma Sm-C concentrations paralleled growth and may regulate lean tissue development as exemplified by relative breast muscle size. It is likely that plasma Sm-C concentrations can be used as an indication of dietary protein adequacy given that energy intake is also considered.

In summary, it is evident that the dietary protein intake *per se* is a potent regulator of avian lipid metabolism; however, assigning a particular rate limiting step in a metabolic pathway to dietary protein alone is difficult. The present study indicates that the dietary protein intake regulates *de novo* fat synthesis. The decrease in lipogenesis associated with an increase in protein intake may be caused by diminished co-factor required for fat synthesis as well as by an increase in the production of glucose from fatty acid precursors. The data in this study also provide an initial basis to assume that somatomedin-C may also be indirectly responsible for a decrease in body fat through a stimulation of lean tissue synthesis.

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